Right ventricular pressure response to exercise in congenital heart septal defects

by

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List of Papers

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Submitted

Möller T, Leren T, Eiklid K, Holmstrøm H, Fredriksen PM, Thaulow E
A novel BMPR-2 gene mutation associated with exercise-induced pulmonary hypertension in cardiac septal defects
Submitted

Abbreviations

ALK1	activin receptor-like kinase 1
ASD	atrial septal defect
BMPR-2	bone morphogenetic protein receptor type 2
ECG	electrocardiogram
ENG	endoglin
ExE	exercise echocardiography
HHT	hereditary hemorrhagic teleangiectasia,
	synonym: Morbus Osler, Osler-Weber-Rendu syndrome
mPAP	mean pulmonary arterial pressure
PAH	pulmonary arterial hypertension
PVR	pulmonary vascular resistance
RV	right ventricle
RVOTO	right ventricular outflow tract obstruction
RVPR	right ventricular systolic pressure response
RVSP	right ventricular systolic pressure
sPAP	systolic pulmonary arterial pressure
SpO_2	peripheral oxygen saturation
TAPSE	tricuspid annular plane systolic excursion
TASM	peak tricuspid annular systolic motion velocity
TGF-beta	transforming growth factor-beta
VO_{2peak}	peak oxygen uptake
VSD	ventricular septal defect

1. Introduction

1.1 A case history

E. was born in 1996 as a son of healthy non-related parents. Shortly after birth he developed symptoms of heart failure and a large perimembraneous ventricular septal defect (VSD) was found. With conventional heart failure medication (ACE-inhibitor, furosemide and digoxin) he did well until his VSD was closed surgically at the age of 24 months. He recovered quickly and there was no indication of any residual defect. Postoperatively slightly elevated pulmonary arterial pressure was normalized a few months later. Clinical follow-up was terminated 15 months after defect closure and the boy's parents were informed that their son now was healthy.

For more than a decade the boy developed normally without cardiac symptoms. In summer 2009 he suddenly complained about exercise intolerance with breathlessness and dizziness during soccer training and matches. After several months of medical investigation by his family practitioner he was referred to a pediatric cardiologist. Cardiac examination revealed elevated right ventricular pressure at rest without right ventricular outflow tract obstruction. Based on these findings pulmonary arterial hypertension (PAH) was suspected and the patient was referred to Oslo University Hospital Rikshospitalet for further investigation.

Clinical examination was without abnormal findings, the electrocardiogram (ECG) showed signs of right ventricular strain. Spirometry and CT scan of the lungs were normal. During cardiopulmonary exercise testing on a treadmill the boy had a maximal oxygen uptake of only 40% of expected aerobic capacity in a boy of his age. Echocardiography demonstrated no residual VSD. A velocity exceeding 4 m/s in the tricuspid valve regurgitation jet indicated a systolic pressure of at least 70 mmHg in the pulmonary artery. Right heart catheterization confirmed pulmonary hypertension with almost systemic pressure level in the pulmonary arterial system and with minimal effect of inhaled nitric oxide or 100% oxygen. Genetical analysis of the boy's BMPR-2 gene by DNA sequencing and by multiplex ligation-dependent probe amplification (see chapter 3.10) revealed no mutation or insertion/deletion of the gene. Medication of his PAH has recently been established.

1.2 Growing up with congenital heart disease

Advances in cardiac surgery and improved survival during the last few decades have created a new and rapidly increasing patient group for adult cardiology services to deal with: adolescents and adult patients with congenital heart disease of varying complexity and often with a history of multiple previous surgical interventions (1). Common synonyms for these patients are GUCH ("grown-up with congenital heart disease") and ACHD ("adult with congenital heart disease"). Major concerns of clinical medicine and research are quality of life and long-term preservation of physical fitness in these patients as they often have residual defects or circulatory problems due to palliative surgery.

1.2.1 Exercise capacity in congenital heart disease

Exercise capacity in adult patients with congenital heart disease in general is reduced and it declines by age almost independently of the character of the initial defect (2;3). The causes and mechanisms of reduced exercise performance in these patients are not well understood. This patient group is heterogeneous in terms of cardiac anatomy and function which makes it difficult to identify and study single elements of cardiac and circulatory function that could cause impaired exercise performance. Strong and valid tools to assess left ventricular performance non-invasively by Doppler echocardiography have been developed for use in conventional cardiology and structural normal hearts. However, the right ventricle and the pulmonary circulation are regularly and strongly affected by various congenital heart defects. These parts of the heart and the circulation are far more difficult to assess reliably by ultrasound techniques. Therefore our knowledge about functional limitations of the right heart and the pulmonary circulation in congenital heart disease is still incomplete.

1.2.2 The understanding of congenital heart disease

The genetical background of congenital heart disease is almost undisclosed compared to our present genetical understanding of i.e. inborn errors of metabolism. Only a few genetical syndromes like trisomy 21 or 22q11-deletion syndrome have related heart malformations caused by a known genetical change. All other congenital heart malformations are caused by genetics which we do not know yet. It would be naive to believe that genetical changes could disturb cell migration during cardiac development leading to different macroscopic malformations without affecting microscopic structures and cellular function. Thus,

repairing the macroscopic structure does not repair the microscopic and functional changes in the congenital malformed heart. These changes continue to affect cardiac and circulatory function after macroscopic defect repair. Therefore it is crucial to achieve an understanding of congenital heart disease as a condition affecting all levels of the circulatory system from macroscopic structures like valves and vessels down to cellular function and intracellular regulatory mechanisms.

1.3 Heart septal defects

Ventricular septal defect and atrial septal defect (ASD) are the second and third most frequent congenital heart defects second after bicuspid aortic valve (4;5). Approximately two/one individuals out of 1000 live births have a VSD/ASD that will not close spontaneously later on. By current clinical practice surgical or catheter-based defect closure is performed in 22% and 67% of cases with non spontaneously closed VSD or ASD respectively (4). Thus, late morbidity related to the heart defect itself or to defect closure is a clinical issue concerning a great number of patients.

The left-to-right shunt of blood flow in ASD and VSD, and eventually the pressure equalisation between the heart chambers in a large VSD, may lead to volume overload or combined pressure and volume overload in the pulmonary vascular system respectively. Pressure and volume cause endothelial stretch that in turn leads to pulmonary vasculopathy and eventually results in PAH (6). Defect closure has been performed in those septal defects assumed hemodynamically significant in order to prevent pulmonary vasculopathy and subsequent PAH. However, occurrence of PAH has been reported in patients with small open septal defects as well as many years after surgical defect closure, even without indications of perioperative PAH (7). The European Heart Survey on Congenital Heart Disease reported a prevalence of secondary PAH to be 18% in closed ASD and 26% in closed VSD (8), mainly from specialized centres. The material consisted of adult patients with late corrective surgery in the 60’s, 70’s and early 80’s compared to current surgical standards. The worrying results of the Euro Heart Survey were presented in 2004. The high prevalence of PAH in this data motivated us to perform this population-based study.

To our knowledge, no data have been presented on the prevalence of PAH in patients with single ASD or VSD with access to cardiac surgery early in life. Otterstad et al published data from a partly invasive study on pulmonary arterial pressure at rest and during mild exercise in adult patients with isolated VSD almost three decades ago (9;10). The authors

demonstrated both reduced exercise performance and a high percentage of abnormal pulmonary arterial pressure response to exercise in their patients. However, none of the patients with surgically closed had been operated before 10 years of age which makes it difficult to compare their results directly to current patients with VSD and early surgical intervention. A better long-term prognosis in terms of lower prevalence of PAH has to be hypothesised when the defect has been closed within the first few years of life as in most cases today.

1.4 Pulmonary arterial hypertension

1.4.1 Definition and classification

PAH has been in particular focus for research for the last twenty years. Major advances have been made in molecular pathobiology and in therapy of PAH. But still there is no generally accepted hemodynamic definition of PAH (11). Several international consensus conferences have suggested and revised the commonly used definition criteria for PAH. The latest revision was made in 2008 on the 4th World Symposium on Pulmonary Hypertension in Dana Point, CA (12). PAH is defined by mean pulmonary arterial pressure (mPAP) $\geq$ 25 mmHg at rest in the presence of a pulmonary capillary wedge pressure $\leq$ 15 mmHg indicating normal pressure conditions in the left atrium. Remarkably the previous criteria for PAH during exercise was removed in the latest revision because of data indicating higher upper normal limits of mPAP during exercise in individuals older than 50 years (12). Likewise pulmonary vascular resistance (PVR) measured invasively is no longer suitable to define PAH in contrast to older definitions.

The clinical definition and diagnosis of PAH has been dependent exclusively on invasive pressure measurement during right heart catheterisation. The development of Doppler echocardiography has become a tempting non-invasive alternative in clinical diagnostics. Comparative studies have shown that echocardiography underestimates pulmonary arterial pressure compared to invasive techniques especially in the presence of high pulmonary pressure (13). Upper normal limits of systolic pulmonary arterial pressure (sPAP) at rest assessed by echocardiography have been defined (14). The upper normal limit of sPAP during exercise is less well documented. Exercise studies in healthy individuals indicate that endurance trained athletes with high cardiac output may show pulmonary pressure conditions during exercise exceeding any definition of normal upper limit (15). Despite

methodological weaknesses Doppler echocardiography remains the best available clinical screening tool in detecting PAH (12;16).

The latest international classification of pulmonary hypertension was published in 2009 based on the consensus from the 4th World Symposium on Pulmonary Hypertension (17).

Updated clinical classification of pulmonary hypertension (Dana Point, 2008):

1. Pulmonary arterial hypertension (PAH)
    - 1.1. Idiopathic PAH
    - 1.2. Heritable
        - 1.2.1. BMPR-2
        - 1.2.2. ALK1, endoglin (with or without hereditary hemorrhagic telangiectasia)
        - 1.2.3. Unknown
    - 1.3. Drug- and toxin-induced
    - 1.4. Associated with
        - 1.4.1. Connective tissue diseases
        - 1.4.2. HIV infection
        - 1.4.3. Portal hypertension
        - 1.4.4. Congenital heart diseases
        - 1.4.5. Schistosomiasis
        - 1.4.6. Chronic hemolytic anemia
    - 1.5 Persistent pulmonary hypertension of the newborn
    - 1.6 Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis

2. Pulmonary hypertension owing to left heart disease
    - 2.1. Systolic dysfunction
    - 2.2. Diastolic dysfunction
    - 2.3. Valvular disease
3. Pulmonary hypertension owing to lung diseases and/or hypoxia
    - 3.1. Chronic obstructive pulmonary disease
    - 3.2. Interstitial lung disease
    - 3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern
    - 3.4. Sleep-disordered breathing
    - 3.5. Alveolar hypoventilation disorders
    - 3.6. Chronic exposure to high altitude
    - 3.7. Developmental abnormalities
4. Chronic thromboembolic pulmonary hypertension
5. Pulmonary hypertension with unclear multifactorial mechanisms
    - 5.1. Hematologic disorders: myeloproliferative disorders, splenectomy
    - 5.2. Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis: lymphangioleiomyomatosis, neurofibromatosis, vasculitis
    - 5.3. Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
    - 5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

The present project deals with the entity of PAH (PAH-classification category 1), where the pathogenesis is located on the arterial side of the pulmonary vascular bed caused by congenital heart disease (PAH-classification category 1.4.4). The classification has been and will be revised due to improving insight into pathogenesis and intrinsic factors of

susceptibility to PAH. In the future it may lead to further differentiation of PAH forms or fusion of different forms with common genetic and pathobiological background yet to discover. The 2008 classification suggest a novel subclassification of PAH secondary to congenital heart disease (category 1.4.4) into (17):

A: Eisenmenger syndrome

B: PAH associated with systemic-to-pulmonary shunts

C: PAH with small defects

D: PAH after corrective cardiac surgery

1.4.2 Pathobiology

Different forms of PAH show a similar molecular and histopathological development of vasculopathy (18;19). In the earliest stage of PAH there are only functional changes with failure of the pulmonary arterioles to dilate. Later in the pathogenetic process increasing muscularisation and wall thickening of the distal arterioles increase pulmonary vascular resistance. Subsequent hyperplasia of intima, media and adventitia leads to vessel occlusion. In the end stage of the disease one can find so-called plexiform lesions, a product of uncontrolled growth of all layers of the arteriolar wall into nodules of chaotic tissue arrangement. It is known that the pulmonal vascular endothelium plays a key role in the pathogenesis. However, the complex interaction between different cell types, signalling pathways, genetic changes and external damaging factors in the pathogenesis of PAH are still incompletely understood.

Several biomarkers are connected to the cellular and molecular pathobiology of PAH. As an important component of the extracellular matrix, fibronectin is known to contribute in cell adhesion, migration, transformation and motility. It modulates phenotype and growth of vascular smooth muscle cells (20). A mechanism for translating cyclic stretch of the vascular smooth muscle cells into increased fibronectin production has been demonstrated in rats (21). In congenital systemic to pulmonary shunts, the migration from media to intima of vascular smooth muscle cells in pulmonary microvessels during pulmonary hyperflow is thought to be driven by a fibronectin gradient (22). Although measured in the circulation and not in the lungs, a higher serum level in patients with pathological pulmonary artery

pressure response during exercise could indicate that the demonstrated abnormal pressure response represents an ongoing vascular remodelling process.

Von Willebrand factor is a marker of endothelial activation. Patients with serious pulmonary hypertension due to congenital heart disease are known to have alterations in von Willebrand factor function (23). A great number of other biomarkers and signalling substances have been investigated and related to different forms of PAH. An increased level of von Willebrand factor in the patients with operated, and thus hemodynamically significant, defects may indicate that these patients show a lasting state of endothelial activation.

The whole aspect of current pathobiological understanding is too complicated to be presented or discussed in this context (24-26).

1.4.3 Genetics

The transforming growth factor-beta (TGF-beta) signalling pathway is crucial in both functional and structural changes in PAH (27). The majority of genes, that have been identified over the last decade and that are associated with different forms of PAH, are coding proteins of the TGF-beta superfamily:

- Bone morphogenetic protein receptor type 2 (BMPR-2) is part of a membrane receptor interfering with cellular hyperplasia and apoptosis. The gene has been identified ten years ago (28-30). BMPR-2 mutations have been found in hereditary PAH in 70% of cases and in idiopathic PAH in 20% of cases (31-34). The prevalence of BMPR2-mutations in the healthy general population is estimated to be approximately 0.01-0,001% (35).

- Activin-like kinase-type 1 (ALK1) and

- Endoglin (ENG) are two TGF-beta proteins associated with a rare form of PAH. Mutations in the ALK1 and the ENG gene have been demonstrated in cases of secondary PAH in hereditary hemorrhagic teleangiectasia (HHT) (36;37).

Interestingly proteins of the transforming growth factor-β (TGF-β) signalling pathway have also been shown to be important prenatally in cardiac and pulmonary vascular development (38-41). Bone morphogenetic protein type 2 (BMP2) is crucial in coordinating multiple aspects of atrioventricular canal morphogenesis (42) and cardiac looping (43). This genetic

connection between PAH pathogenesis and cardiac and pulmonary vascular embryology made genetic changes in the TGF-beta superfamily a key issue to investigate in our study.

1.4.4 Exercise-induced pulmonary arterial hypertension

Exercise-induced PAH or abnormal right ventricular systolic pressure response is an incompletely understood phenomenon and its clinical significance is still uncertain (44). Possible pathophysiological mechanisms behind excessive pressure rise in the pulmonary vascular system during exercise include fixed structural changes in the pulmonary vasculature, pulmonary arteriolar vasoconstriction or disturbances in left ventricular diastolic or systolic function. In patients with congenital heart disease, post-surgical alterations in contractility like dyssynchrony caused by left bundle branch block may contribute to a backward failure resulting in exercise-induced PAH (45).

Exercise-induced PAH is a marker of early pulmonary vasculopathy in hereditary PAH and in PAH associated with scleroderma in individuals who will develop PAH at rest later in life (44;46;47). Exercise-induced PAH or abnormal right ventricular systolic pressure response (RVPR) during sea level exercise and RVPR due to hypoxia has been shown to correlate closely. Both methods can identify individuals susceptible to high altitude pulmonary oedema (48-50) which demonstrates the common pathophysiological vasoconstrictive nature of hypoxic PAH and exercise-induced PAH.

The pathogenesis of PAH beginning with functional disturbances progressing into structural changes gives rise to the hypothesis of exercise-induced PAH as an early stage of disease in any form of PAH. However, this progression from early exercise-induced PAH into PAH at rest has to be demonstrated in a longitudinal approach for each of the different etiological forms of PAH like PAH secondary to congenital heart disease with left-to-right shunt.

2. Aims of the study

2.1 Exploration of methods and normal limits

We aimed to define the normal range, in particular the upper normal limit, of right ventricular systolic pressure (RVSP) in adolescents and young adults during exercise, as measured by non-invasive ultrasonic techniques. We aimed to investigate the interrelation of RVSP to the aerobic exercise capacity, and to establish whether endurance-trained athletes differ from the normally trained population.

2.2 Prevalence of exercise-induced PAH

In this main part of our study we wanted to investigate right ventricular pressure response (RVPR) to exercise and its interrelation to aerobic capacity and to right ventricular (RV) performance. We aimed to test our hypothesis that exercise-induced would be found more often in patients with large defects, late defect closure and more often in VSD than ASD.

2.3 Characterising the condition: dynamic versus static

The altitude study aimed to explore to what extent abnormal RVPR in patients with surgically closed ASD or VSD is a dynamic condition which can be influenced by external factors like hypobaric hypoxia during simulated moderate altitude conditions. We wanted to investigate if abnormal RVPR can be evoked by moderate altitude or if abnormal response at sea level can be worsened by moderate altitude. We were interested whether altitude-induced changes likewise would be more pronounced in patients with VSD as compared to patients with ASD who had shown lower prevalence of exercise-induced PAH (51).

2.4 Genetical susceptibility

Finally we aimed to investigate the relationship between exercise-induced PAH in our group of patients and genetic changes related to the TGF-β signalling pathway.

3. Material and methods

3.1 General methodological considerations

Careful consideration of examination methods is necessary for investigation of pulmonary circulation. Non-invasive techniques like echocardiography and cardio-pulmonary exercise testing are preferable by ethical considerations and they are relatively inexpensive. Invasive measurements during right heart catheterisation are more precise and reliable but by today's clinical resources and ethical standards they are almost impossible to achieve in large study groups of patients and healthy controls.

Non-invasive techniques permit examination of numerous individuals without the need of invasive heart catheterisation. Ultrasound measurements reliably assess many structural and functional parameters inside the heart and the great vessels. In almost every echocardiographic measurement there is a varying degree of observer dependent variability and accuracy. Both over- and underestimation are relevant issues. I.e. the overestimation of pressure gradients by Doppler techniques compared to catheter-based measurements is well known. Thus, the researcher, who wants to investigate a hemodynamic phenomenon like exercise-induced PAH, has to face technical limitations and questions about data quality. As the aim of the study was to investigate the prevalence and character of exercise-induced PAH in a large group of patients and healthy controls we had to choose a non-invasive approach.

Exercise echocardiography had its first breakthrough in research during the 1980's. Several issues were studied by this practical challenging technique: contractility in heart failure or coronary vessel disease and pressure gradients in valve disease were the main fields of investigation. As the use of pharmacological stress during echocardiography became more widespread, the need to compromise with poor image quality and exercise derived artefacts was less attractive.

The ability of exercise echocardiography to detect exercise-induced PAH has been demonstrated in patients with scleroderma (47), in individuals with a genetic risk of PAH (46;52) and in healthy individuals susceptible to high altitude pulmonary oedema (53). Exercise echocardiography has also been performed in small patient groups with congenital heart disease (54-57).

Echocardiography has been shown to give reliable measurements of RVSP compared to invasive measurement at rest (58) and during exercise (54;59). In the absence of right ventricular outflow tract obstruction, RVSP reflects pulmonary arterial pressure (60-62). Previous studies have demonstrated the relationship between systolic and mean pulmonary arterial pressure (63;64) which by consensus defines the diagnosis of PAH (65).

3.2 Patient group

In the Norwegian regions of Vestfold, Asker and Bærum, 72 children were born between 1982 and 1993 with isolated VSD or ASD and without right ventricular outflow tract obstruction (RVOTO). This count did not include patients with spontaneously closed foramen ovale or VSD during follow-up and patients with complicating conditions preventing appropriate exercise testing (i.e. trisomy 21). The 72 patients had at the time of inclusion either an open defect or they had undergone defect closure. Two individuals with device-closed ASD were excluded to prevent device related confounding. Three patients with surgical closed VSD were excluded because of abnormal rise in right ventricular outflow tract velocities above 2.0 m/sec during exercise indicating dynamic RVOTO. Of 67 eligible patients 23 were lost to follow-up (6 patients) or refused participation (17 patients). Among these 23 were 18 patients with VSD and 5 patients with ASD. As these patients were not examined in our study there might have been several cases of spontaneous defect closure after the latest cardiac examination report in their medical record. The patients who were lost to follow-up had either changed their home address and could not be identified in official registries, or did not answer our invitation, or did not meet to any of maximal two clinical appointments. The reasons for refusing participation varied from lack of time, participation in other clinical studies, and to unwillingness to be interviewed and examined in a setting related to their heart condition. We had no indications that there were any cases of current cardiac problems or complications involved in those 23 eligible cases that did not participate.

Finally 44 patients could be included in the study. All patients with ASD had a surgically closed defect, 3 by patch closure and 14 by direct suture. Eleven patients had their VSD closed surgically, 7 by direct suture and 4 by patch closure. Two of these (18%) had minor residual shunts. No patient received specific treatment of PAH before or after surgery; however, preoperative catherization data were available only in a few patients. Velocity measurements across the ventricular septum in 16 patients with untreated small muscular or

small perimembraneous VSD (4.5±0.51 m/s) indicated normal RV pressure at rest. Two defects were identified by color-Doppler mapping but were too small for velocity assessment. For basic patients group characteristics see paper II, table 1.

3.3 Control group

The patient group was matched individually 2:1 against 88 healthy control subjects of the same sex and age (+/- 12 months). The healthy control subjects were recruited among hospital employees and their relatives, among college students and among other local citizens volunteering subsequent to newspaper articles. The volunteers had no known heart or lung disease; however mild bronchial asthma was accepted. For basic control group characteristics see paper II, table 1.

3.4 Clinical examination

All patients and healthy members of the control group were examined including auscultation of the heart and lungs, measuring of resting blood pressure and obtaining an electrocardiogram (ECG). A medical family history and construction of a pedigree was part of the standard interview.

3.5 Exercise testing

All patients and control subjects were examined by cardiovascular exercise testing on a treadmill ergometer with gas exchange analysis and ECG following the Oslo protocol (66) (Equipment: Jaeger Oxycon Delta, VIASYS Healthcare GmbH, Höchberg, Germany). Peak oxygen uptake (VO_{2peak}) was corrected for body weight (67) and expressed as $ml/kg^{-0.67}min^{-1}$. The individual results were compared to reference values from healthy Norwegian adolescents (68) and expressed by standard deviation (Z-score) from age-related mean in the reference material. Highly endurance-trained volunteers with a Z-score > 2 were excluded from the study because of the known phenomenon of elevated pulmonary arterial pressure levels in athletes (15;69). One patient's treadmill exercise was considered submaximal and consequently excluded from further analysis.

We chose to perform a maximal cardiopulmonary exercise test before exercise echocardiography even if this order of tests could reduce pulmonary vascular resistance and lead to falsely low exercise-induced RVSP values. We tried to minimise the effect by having a resting phase of one hour between treadmill exercise and supine cycling during exercise echocardiography. The alternative of performing the tests on different days would

have been a difficult task for most participants who mainly had educational obligations as students. Measurement of oxygen consumption during supine cycling as a second alternative would have lead to falsely low VO_{2peak} results (70).

3.6 Echocardiography at rest

Echocardiographic recordings were obtained with a Vivid 7 scanner (GE Vingmed Ultrasound, Horten, Norway). All echocardiographic studies were videotaped and saved digitally (still frame and loops). Right atrial pressure at rest was estimated by vena cava inferior index (71). RV performance (72-74) was measured by M-mode registration of tricuspid annular plane systolic excursion (TAPSE) (75) and peak tricuspid annular plane systolic motion velocity (TASM) measured by colour tissue Doppler (76;77).

3.7 Exercise echocardiography

One hour after treadmill testing, exercise echocardiography was performed during supine cycling with about 30 degrees elevation and left side tilt (Equipment: Ergoselect 1200 EL, Ergoline GmbH, Bitz, Germany). A stepwise exercise protocol was used with a starting load of 25 Watt and an increase of 25 Watt every second minute until the target heart rate of 160-min was reached. Above that level, echocardiographic recordings become futile because of upper body movement and interposition of the lungs. Systemic blood pressure was measured at every exercise level, as well as the maximal velocity of tricuspid regurgitation jet. The right ventricular systolic pressure (RVSP) was calculated from each recording by means of the modified Bernoulli's equation, adding the right atrial pressure at rest to the calculated pressure gradient between RV and right atrium (58). In order to detect dynamic RV outflow tract obstruction that could interfere with RVSP measurements, RV outflow tract velocity was measured at the 100 Watt level. Patients with outflow tract velocities higher than 2 meters per second were not included in the study.

3.8 Altitude simulation

The selected patients for the altitude study were examined by ExE during supine cycling at sea level right before exposition to altitude conditions. Using a hypobaric chamber the patients then were exposed to a simulated ascent to an altitude of 2500 meters or 8200 feet above sea level. During the following two hours of supine rest (altitude adaptation phase) peripheral oxygen saturation (SpO_2), blood pressure, RV performance and RVSP were monitored every 15 minutes. Finally ExE was repeated before descent.

All altitude tests were conducted in a hypobaric chamber (Norwegian Universal Technology AS, Haugesund, Norway). After decompression atmospheric conditions were monitored every five minutes. During altitude adaptation and altitude exercise the air temperature was 21.5±1.2 ºC. Chamber pressure after decompression simulated a stable altitude of 2500 meters, oxygen percentage was 20.8±0.04 % and carbon dioxide percentage was 0.044±0.013 %.

3.9 Biochemical analysis

Blood samples were taken of all patients prior to exercise for biochemical analysis. Concentration of haemoglobin, sodium, potassium and N-terminal pro brain natriuretic peptide (pro-BNP, kit Modular E by Roche Diagnostics AS, Oslo, Norway) were assessed. Fibronectin was measured by EIA using antibodies from DakoCytomation (Denmark). EIAs for CRP, vWf, IL-8, sTNFR1, TNFα, MCP-1, RANTES, OPG and CD40-ligand.were performed as formerly described (78). Briefly, 96 well plates were coated overnight at 4ºC with 100 uL rabbit anti-human antibody at 10 mg/L in PBS. 0.1% Tween 20 in PBS was used as a buffer in subsequent steps. Standard was pooled serum diluted 1:100 – 1:6400. After blocking samples, 100 uL standard or diluted samples (1:1600) were added and incubated at 37C for 2 hours. The plate was washed and 100 uL peroxidase-conjugated rabbit anti-human antibody was added (1:4000) and incubated 37C for 1 hour. Plates were developed with tetramethylbenzadine (Zymed Laboratories Inc., Germany), stopped with H_2SO_4, and read at 450 nm. All samples from the same individual were run on the same plate to avoid run-to-run variation. All patients with abnormal RVPR had VSD (Paper II). We therefore analyzed for differences between those surgically treated and those not, due to the fact that surgical intervention is an indicator of former hemodynamic significance of the lesion.

3.10 Genetical analysis

Screening for mutations in the BMPR-2 gene (GenBank accession number Z48923.1) was performed by DNA sequencing to detect point mutations and small insertions/deletions within exons and the flanking intron sequences, and by multiplex ligation-dependent probe amplification (MLPA) to detect structural alterations. For DNA sequencing, DNA was extracted from EDTA-containing blood by the use of a BioRobot EZ1 (Qiagen GmbH, Hilden, Germany). Individual exons with flanking intron sequences were amplified by PCR. Typically, approximately 50 base pairs of flanking intron sequences were included in the

amplicons. The primer sequences and conditions for the thermal cyclings are available upon request. Standard DNA sequencing reactions using version 3.1 of Big Dye terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) were analysed on a Genetic Analyzer 3730 (Applied Biosystems Inc., Foster City, CA). The software prediction program PolyPhen (www.bork.embl-heidelberg.de/PolyPhen/) was used to assess the pathogenicity of identified missense mutations. For MLPA analysis, DNA was extracted from EDTA-containing white blood lymphocytes by the manufacturer's protocol, on a Magnapure DNA Extractor (Roche Diagnostics GmbH, Mannheim, Germany). The SALSA MLPA kit P093 HHT/PPHT1 (MCR-Holland, Amsterdam, The Netherlands) was used to test for deletions or duplication in the BMRP-2 gene, the ENG gene and the ALK1 gene.

4. Summary of results

4.1 Paper I and related results

Our data has shown a great variability in right ventricular pressure response to exercise. There is no linear correlation between maximal right ventricular systolic pressure and aerobic capacity, but athletes with high aerobic capacity often show an abnormally high RVPR. The upper normal limit of the RVPR in normally trained individuals seems to be 50 mmHg RVSP which is higher than commonly assumed (79).

Athletes show a continuous rise in RVSP without ever reaching a plateau even during high workload levels presumably due to extremely high cardiac output as shown in previous studies (15;69;80). As both normal and abnormal RVSP responders in our study reach a RVSP plateau during exercise one would assume different mechanisms leading to high RVSP pressure during exercise in these patients and in highly trained athletes.

4.1.1 Inter-observer agreement and variability of exercise echocardiography

For analysis of inter-observer variability a blinded second analyzer evaluated 35 ExE recordings (18 patients, 17 controls). The second analyzer was experienced in ExE technique. Inter-observer agreement was expressed by a Bland-Altman-plot (81).

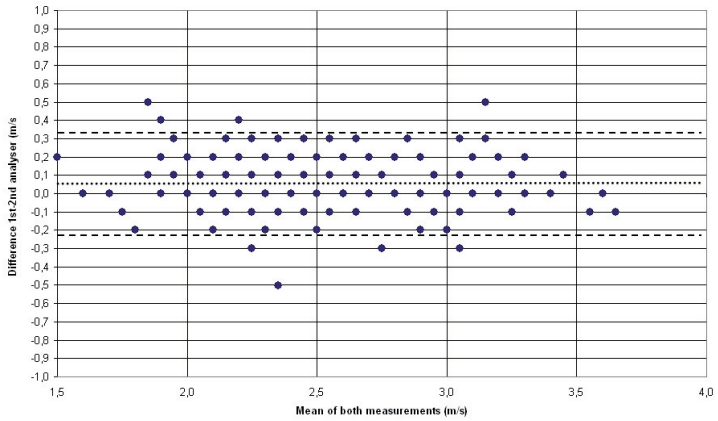

Figure 1: Bland-Altman-Plot of inter-observer variability in tricuspid regurgitation jet velocity measurements

The two independent observer's analysis showed very good agreement. The 95 % limits of agreement were -0.17/0.33 meters per second with a positive bias of only 0.06 meters per second in Doppler velocity measurements (figure 1) and only 2 mmHg in maximal RVSP between first and second analyzer. There was no difference in agreement depending on low or high velocities. For tricuspid regurgitation velocities measured offline by the two observers, the intraclass correlation coefficient was 0.93 ($p<0.001$). Inter-observer agreement was possible to measure by Kappa statistics in order to define abnormal pressure response to exercise. With a cut-off at 50 mmHg RVSP, Kappa was 0.82. Our study results from inter-observer agreement have not been published elsewhere.

4.2 Paper II and related results

4.2.1 Aerobic exercise capacity and RV performance

Aerobic exercise capacity expressed by Z-score of VO2peak in the patient group and subgroups were significantly lower compared to the control group (paper II, table 2, figure 4). There was no difference in aerobic capacity between any of the different patient groups (ASD, all VSD, open VSD, closed VSD). Z-score of VO2peak correlated inversely to age at defect closure for VSD but not for ASD. The patients who had undergone surgical defect closure had significantly reduced RV performance with no difference between closed ASD and closed VSD (paper II, table 2, figure 5).

4.2.2 Exercise-induced pulmonary hypertension

There was no case of pulmonary hypertension at rest (RVSP > 40 mmHg) and no difference in RVSP at rest between patients and control group. Exercise echocardiography permitted assessable measurements of RVSP by tricuspid regurgitation jet velocity in 85 controls (97 %) and in all patients. The difference between the patient group and the control group during exercise was not evident in the group mean of maximal RVSP (paper II, table 2, figure 6) but in pressure rise pattern (paper II, figure 1) and in the frequency of abnormal RVSP response (paper II, table 3). There was a higher rise angle in RVSP during the first minutes of incremental workload in the patient group compared to the control group ($p=0.007$). The number of subjects with RVSP response > 50 mmHg was significantly higher in both VSD groups compared to the ASD group ($p=0.044$ in open VSD versus ASD, $p=0.005$ in closed VSD versus ASD). There was no difference in the occurrence of

abnormal RVSP response > 50 mmHg between patients with untreated small restrictive VSD and surgically closed VSD (4/16 and 5/11 respectively) and no relationship between abnormal RVSP response and residual VSD after surgery. However, in contrast to the subgroup with closed VSD the higher frequency of abnormal RVSP response within the subgroup of patients with untreated small VSD compared to the matched control subgroup did not reach statistical significance (paper II, table 3). The curve angle of RVSP rise in patients with RVPR > 50 mmHg was steeper than in other patients ($p<0.001$) (paper II, figure 2). Normal and abnormal RVSP responders reached a RVSP plateau at different pressure levels. Systolic blood pressure rise during the first minutes of exercise showed no difference in curve angle in patients with normal or abnormal RVPR (paper II, figure 3).

4.2.3 Intracardiac dyssynchrony / QRS-prolongation

Resting electrocardiogram showed no significant difference in occurrence of QRS prolongation among patients (3/44, 7%, all post surgery VSD) and controls (2/88, 2%). There was a significant correlation between QRS prolongation in the resting electrocardiogram and abnormal RVPR above 50 mmHg for the control group (QRS prolongation in 1/4 abnormal responders, $p=0.002$) but not for the patient group (QRS prolongation in 1/9 abnormal responders).

4.2.4 Results of biochemical analysis

All standard measurements of haemoglobin, sodium, potassium were within normal range and without relationship to RVPR or aerobic exercise capacity. Pro-BNP was measured in 42 patients. Two patients had pro-BNP values above the upper normal limit of 20 pmol/L (24 and 31 pmol/L respectively). No significant correlation was found between pro-BNP exceeding 20 pmol/L and abnormal RVPR.

For the whole patient group (n=38 with available s-fibronectin data), a weak connection was found between maximal right ventricular pressure during exercise and serum fibronectin levels (Spearman's Rho = 0.28, $p=0.095$) (figure 2). The group of patients with maximal RVSP > 50mmHg (n=8) showed a tendency towards a higher serum fibronectin level than in patients with maximal RVSP ≤ 50mmHg (mean 180 µmol/mL [112, 248] versus 127 µmol/mL [105, 151], $p=0.056$) (figure 3). Patients operated for VSD had higher von Willebrand factor than those not operated (123 µmol/mL [91,156] versus 87 µmol/mL [66,109], $p=0.03$) (n=23 with available von Willebrand factor data).

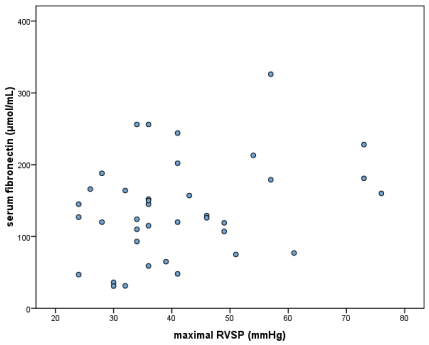

Figure 2: scatterplot of s-fibronectin versus maximal RVSP during exercise.

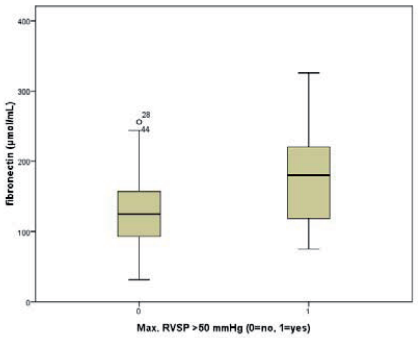

Figure 3: boxplot of s-fibronectin in 30 patients with RVSP response within normal limits as compared to 8 patients with abnormal RVSP response.

Results from biochemical analysis have not been published elsewhere.

4.3 Paper III

Moderate altitude increases oxygen desaturation during exercise and right ventricular systolic pressure at rest and during exercise in adolescents with ventricular or atrial septal defects. Oxygen desaturation during altitude exercise is more pronounced as compared with data from studies in healthy individuals (82-84). RVSP may increase by exposure to moderate altitude alone. Abnormal RVPR to exercise can be evoked by moderate altitude in individuals with normal RVPR at sea level. Abnormal RVPR to exercise at sea level can be aggravated by moderate altitude. Our study did not demonstrate any relationship between aerobic exercise capacity and changes in RVPR or oxygen

4.4 Paper IV and related results

We examined the same group of 44 subjects with isolated ASD or VSD as presented in paper I and paper II. Two patients with borderline or abnormal RVSP response to submaximal exercise were found to be heterozygous for mutation Y589C (c.1766, A>G) in exon 12 of the BMPR-2 gene and three were found to be heterozygous for mutation S775N (c. 2324, G>A) in exon 12 in the same gene. Analysis of the pathogenicity of two mutations

by the use of the prediction program PolyPhen, revealed that mutation Y589C was predicted to be “Probably damaging”, whereas S775N was predicted to be “Benign”. None of the subjects had deletions or duplication in the BMPR-2 gene, the ALK1 gene or the ENG gene.

To our knowledge mutation Y589C in the BMPR-2 gene is a novel mutation predicted by PolyPhen to be pathogenic, whereas mutation S775N has been previously reported (85) and has also been found in healthy controls (86).

4.4.1 Pedigree analysis

For all included patients and control subjects a pedigree of three generations was obtained. Of all subjects there was only one family with multiple cases of congenital heart defect. It was the family of a 14 year old girl with a minor untreated VSD in the muscular septum. She had five siblings of which one had Tetralogy of Fallot and another was born with a muscular VSD that closed spontaneously during childhood. The girl from our study had a normal RVSP response during exercise and no detectable DNA mutation.

None of the other 131 pedigrees showed any familial recurrence of congenital heart defect or individual medical histories indicating pulmonary hypertension. Pedigree results have not been part of any publication yet.

5. General discussion

5.1 Aerobic exercise capacity

The findings of reduced aerobic capacity in all patient groups confirm previous studies (2;3;54;55) though without any indication that abnormal pulmonary pressure response limits exercise capacity. Interestingly we can demonstrate a positive effect towards better exercise capacity when patients with VSD had their defect closed earlier. As this effect seems not to be related to pulmonary vasculopathy, it may be hypothesised that sternotomy in younger patients is tolerated better with a lower tendency to restrictive respiratory characteristics later in life (87).

5.2 Exercise-induced pulmonary arterial hypertension

Technically our non-invasive investigative approach studies systolic pressure conditions in the right ventricle at rest and during exercise. Hence some conclusions have to be based on previous data and study results like the possibility to estimate right atrial pressure which in addition is assumed nearly unchanged during submaximal exercise. We find the published data on this issue sufficient to allow conclusive RVSP measurements.

Whether measurements of RVSP permit conclusions about pulmonary arterial pressure is another important discussion. The primary source of confounding to be excluded is, of course, any flow obstruction between the right ventricle and the pulmonary arterioles as we have tried to take in account in our study protocol. As PAH is defined by invasively measured parameters like mean pressure and PVR, one could argue that catheterisation would have been the only acceptable way to acquire information about exercise-induced PAH. In the light of ethical considerations and the documented validity and reliability of echocardiographic techniques, we think that our methods and results allow careful conclusions abnormal RVPR reflecting a pulmonary hypertensive phenomenon.

As patients with closed ASD showed a RVSP response to exercise comparable to controls it seems that excessive pulmonary vascular flow without pressure overload is not enough to cause changes in pulmonary vascular endothelium. The patients with closed VSD showed significantly elevated RVPR as a group and a significantly higher rate of abnormal pressure responders. These findings could be interpreted in terms of exclusively pressure-related vasculopathy if not patients with unoperated minor VSD had shown the same pattern of

pathological RVPR to exercise which is a surprising finding. Hypothetically the inadequate ventricular septation itself might be part of a cardiopulmonary vasculopathy that also affects the pulmonary endothelium. The common involvement of TGF-beta signalling in cardiac development, pulmonary vascular development and post-natal pathogenesis of PAH supports this hypothesis. Thus, our data may support the theory of multi-etiological PAH caused by a combination of external triggering factors like pulmonary vascular overload and intrinsic susceptibility that may include male sex and other genetical factors (88).

Results from the altitude study demonstrate that exercise-induced PAH in our patient group can be affected by external conditions. This finding contradicts speculations that exercise-induced PAH only reflects static structural changes in the pulmonary vasculature due to early excessive pulmonary blood flow. Our data merely suggests that exercise-induced PAH reflects a dynamic pulmonary vasoconstrictive mechanism which presumably involves the pulmonary vascular endothelium. This theory is also supported by our finding of elevated inflammation markers in patients with abnormal RVPR.

5.3 Right ventricular performance

Our data show that RV performance reduces in patients who have undergone cardiac surgery. Hypothetically the surgical incision of the pericardium and the right atrium, the mobilisation of the tricuspid valve apparatus, implantation of artificial patches, post-operative inflammation and scarring are possible mechanisms that may influence on right ventricular contractility. Disturbances in the pulmonary circulation like exercise-induced PAH may represent an afterload stress of varying degree that also may reduce RV performance over time. Our data support the common view that RV function plays a key role in long-term functional outcome in congenital heart disease especially after corrective cardiac surgery. Improved techniques of visualisation and functional RV assessment hopefully will give answers to the question about nature of decreased RV performance.

5.4 Clinical consequences

As the average age at defect closure has decreased during the last 20 years, a lower prevalence of abnormal RVPR would be expected in patient populations treated according to modern surgical practice. However, our data could not demonstrate that age at surgery predicts abnormal RVPR later in life. The finding of abnormal RVPR in patients with small unoperated VSD is surprising and warrants further investigation, as these patients often are

outside follow-up programs. A comparable previous study has not demonstrated late presenting PAH in patients with small untreated VSD (89).

Our findings raise some questions about the indications for surgical treatment, timing of the operation and follow-up, but the data do not allow for firm conclusions or guidelines. Further studies are needed in order to define the potential benefit and optimal timing of surgery, especially with respect to small defects. The natural course of abnormal RVPR during exercise is also unknown. In case it represents a dynamic condition with worsening by time, it may warrant follow-up and even medical treatment of these patients. As every second of the patients with closed VSD showed exercise-induced PAH it seems worth to consider life-long clinical follow-up in this group even if the immediate surgical result is good without residual defects.

5.5 Future perspectives

Due to this possible progression into PAH and in the light of the presented data one might consider screening of all VSD patients by exercise echocardiography in adolescence. In case of abnormal RVSP response the individual clinical follow-up should be continued life-long in all patients with treated or untreated open left-to-right shunt regardless defect size. Treatment of possible later PAH at rest then could be initiated before the debut of clinical symptoms which might improve the patients' prognosis.

Our genetical findings might just be early results in a rapidly expanding field of genetical research in congenital heart disease. One might speculate that the near future will bring genetical diagnostics into clinical and surgical decision-making. A patient with genetical determined susceptibility to PAH might be scheduled for earlier defect closure than a patient without a certain mutation. In the future some patients might even receive pulmonary vasodilative treatment in infancy waiting for surgical or catheter-based defect closure, if their genetical risk of pulmonary vasculopathy is high.

5.6 Strength and limitations of the study

The inclusion of patients to the study was population-based as there was no systematical difference between included and not-includable patients from the eligible proportion of all subjects born with isolated VSD or ASD. This strengthens our results and conclusions about the observations. A particular advantage was the ability to identify patients outside clinical follow-up with minor open VSD or with uncomplicated surgically closed defects without

residual defect. These groups are often ignored by publications about long-term morbidity in congenital heart disease as they are difficult to identify without good medical registries and a nationally coordinated public health care system. The mean age at surgical defect closure was somewhat higher in the late 80's and early 90's compared to current standard. Early surgical defect closure did not seem to influence or prevent later exercise-induced PAH at least in the presented study. In our opinion that makes the presented data applicable on today's patient population even if cardiac surgery if performed earlier in life.

In a simplified way, our study allows investigation of three subgroups of different overload situations in the pulmonary circulation that may cause damage in the pulmonary endothelium. Minor pulmonary vascular overload in patients with small open VSD assumed hemodynamically insignificant. These patients have a small transseptal high velocity jet which may affect hemodynamics and endothelial function. Isolated volume overload in patients with a closed ASD and finally combined volume and pressure overload in patients with a closed moderate or large VSD. The last 2 groups have a varying duration of pulmonary vascular shear stress until defect closure. As perioperative PAH was absent in patients with closed defects abnormal RVPR represents rather a closure independent condition than a vasoconstrictive residual after previous PAH.

Our institutions' medical records did unfortunately not allow differentiation of preoperative defects into moderate or large left-to-right shunt as there were no catheterisation data or chest X-ray available in every case. Thus, the hypothesis that the patients with the largest defects preoperatively would show the highest prevalence of exercise-induced PAH later in life could not be tested in our study. It is important to remember that the population-based distribution of defects in our material favouring small and moderate sized defects.

The pro-and-contra of our non-invasive approach in this study has been addressed in section 3.1. In our study we had to examine a large number of healthy young patients and an even larger number of healthy volunteers. This enabled us to draw general conclusions about prevalence of exercise-induced PAH in patients with VSD. By ethical, practical and financial reasons an invasive study design based on right heart catheterisation data would have been impossible to achieve. Thus, performing our investigative task meant to deal with the weaknesses of ultrasound technology. By doing a proper method evaluation and by exploring the limits of normality in RVPR as described in paper I, we wanted to minimise this methodological disadvantage. However, this research strategy could never give answers

about invasive hemodynamic characteristics like pulmonary vascular resistance which is often proposed as the gold standard of PAH diagnostics. The underlying hemodynamical mechanisms of exercise-induced PAH and their relation to i.e. intrapulmonary circulation or left heart dysfunction will be challenging to investigate even with invasive techniques.

6. Conclusions

Exercise-induced pulmonary hypertension can be reliably assessed non-invasively by exercise echocardiography. Exercise-induced PAH has a high prevalence in healthy young patients with VSD. It can be found in patients who had their defect closed early in life, but also in patients with small VSD that are commonly considered hemodynamically benign. Thus, exercise-induced PAH seems to be caused not only by hemodynamic influence of shunt-related excessive blood flow to the pulmonary vascular system before the defect is closed. Our data suggest that mutations in the TGF-beta signalling pathway may play a role in the pathogenesis of exercise-induced PAH in congenital heart malformations in the same way as it has been shown to act in other forms of PAH.

Our data indicate that exercise-induced PAH is not due to a fixed anatomic change in the patients' pulmonary vascular bed. It seems rather to be a dynamic vasoconstrictive condition which can be evoked or aggravated by external factors like hypobaric hypoxia. This finding leads to the hypothesis that there might be a progression into PAH at rest as the patient gets older, at least in some cases. This hypothesis should be the subject of further investigation.

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Paper I – IV

I

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Original Article

Non-invasive measurement of the response of right ventricular pressure to exercise, and its relation to aerobic capacity

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Abstract *Introduction:* Exercise echocardiography assesses exercise-induced pulmonary hypertension. The upper normal limit of right ventricular systolic pressure during exercise is not well established. Our study aims to investigate the response of right ventricular systolic pressure in relation to aerobic capacity. *Methods and results:* Cardiopulmonary exercise testing using a treadmill, and echocardiography during supine cycling, were performed in 113 healthy volunteers aged 13 to 25 years. Maximal right ventricular systolic pressure during evaluable exercise studies obtained in 108 subjects showed a Gaussian distribution only after separating the endurance trained subjects, specifically 12 athletes with Z-score of peak oxygen uptake higher than 2.0, from the normally trained group of 97 subjects. Maximal right ventricular systolic pressure during exercise in the normally trained group showed a mean of 38.0 millimetres of mercury, with standard deviation of 7.2, a median value of 39.0, and a range from 17 to 63, and the 95th percentile was 51 millimetres of mercury. In the athletes, the maximal right ventricular systolic pressure was higher, with a median of 55.5, a range from 28 to 69, this being significant, with p equal to 0.004). Of the 12 athletes, 8 (67%) showed a response of right ventricular systolic pressure to exercise exceeding 50 millimetres of mercury, but only 8 of 97 normally trained subjects (8%) showed a similar response, this also being significant, with p less than 0.001. *Conclusions:* Our study confirms the great variability in the response of right ventricular systolic pressure to exercise in healthy individuals, with 50 millimetres of mercury representing the upper normal limit. Endurance-trained athletes show higher levels, and two-thirds have abnormal responses exceeding 50 millimetres of mercury.

Keywords: Exercise echocardiography; exercise physiology; pulmonary hypertension; pulmonary circulation

Pulmonary arterial systolic pressure, and right ventricular systolic pressure, are approximately one-quarter of systemic arterial pressure, and are considered to remain nearly unchanged during exercise. Few studies, however, have investigated normal pulmonary pressures during exercise.[1–3] A known pulmonary arterial systolic pressure allows calculation of the mean pulmonary arterial pressure, which by consensus defines pulmonary arterial hypertension.[4,5] The use of invasive methods to measure pulmonary arterial pressure in healthy individuals is limited by ethical considerations. Echocardiography has been shown to provide reliable measurements compared to invasive measurement at rest,[6] and during exercise.[7,8] In the absence of an obstructed right ventricular outflow tract, the systolic pressure reflects, but does not equal, pulmonary arterial systolic pressure.[2]

International guidelines define the normal upper limits of pulmonary arterial systolic pressure at rest, but there is a general uncertainty about the upper

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limits for both pulmonary and right ventricular systolic pressures during exercise.[9,10] A value of 40 millimetres of mercury, or in some papers 45 millimetres of mercury, is proposed as the upper normal limit during exercise.[2,3,11] Recent studies, however, have shown an abnormal response of right ventricular systolic pressure above 45 millimetres of mercury during exercise in healthy, normal individuals,[12] and also in healthy carriers of mutations in the bone morphogenetic protein receptor type 2 gene.[13] Such abnormal responses have been shown to be a marker of susceptibility to high altitude pulmonary oedema.[14] Some investigators have also shown abnormal responses in endurance-trained professional athletes,[15] whereas others demonstrated a normal response.[3] It has been hypothesized that the extremely high cardiac output of such athletes exceeds their pulmonary vascular dilative capacity, and thereby may lead to pulmonary vascular pressures above normal limits.[1,15]

The pulmonary endothelium is adapted to conditions of low blood pressure. Endothelial damage is well known in different kinds of pulmonary arterial hypertension. In patients with Eisenmenger syndrome, who have untreated or complicated congenital cardiac malformations, shunting of blood from the systemic to the pulmonary circulations results in excessive pulmonary flow and endothelial sheer stress.[16]

We are not aware of any major studies on the normal range of either right ventricular or pulmonary arterial systolic pressures during exercise in healthy adolescents, including non-professional high performing athletes. Exercise echocardiography is of increasing importance as a diagnostic tool in pulmonary hypertension, and normal values of right ventricular systolic pressure during exercise are strongly needed. In this study, therefore, we aimed to investigate the normal range, in particular the upper normal limit, of right ventricular systolic pressure in adolescents and young adults during exercise, as measured by non-invasive ultrasonic techniques. Additionally, we aimed to investigate the interrelation of right ventricular systolic pressure to the aerobic exercise capacity, and to establish whether endurance-trained athletes differ from the normally trained population.

Materials and methods

Population

Healthy volunteers were recruited from hospital employees and their relatives, among college students of a nearby institution, and among local inhabitants. The volunteers were aged between 13 and 25 years, because an age- and gender-matched control group was needed at ratios of 2 to 1 for research in a young population of 45 subjects with congenital cardiac disease. The volunteers had no known heart or lung disease, albeit that mild bronchial asthma was accepted. Detection of any cardiac condition, or any serious lung condition, during the study led to exclusion.

Examination

The clinical investigation consisted of cardiopulmonary exercise testing on a treadmill, along with electrocardiography and echocardiography at rest and during exercise on a supine cycle ergometer. The participants performed a maximal exercise test with gas exchange analysis and electrocardiogram on a treadmill ergometer following the Oslo protocol[17] (Equipment: Jaeger Oxycon Delta, VIASYS Healthcare, Höchberg, Germany). Peak oxygen uptake was corrected for body weight[18] and expressed as $\mathrm{ml\,kg^{-0.67}\,min^{-1}}$.

The individual results were compared to reference values from healthy Norwegian adolescents,[19] and expressed by standard deviation, the Z-score, from the age-related mean in the reference material. Highly endurance trained individuals with a Z-score above 2.0 were defined as athletes. The others, with Z-scores equal or less than 2.0, were defined as normally trained subjects.

All echocardiographic recordings were obtained with a Vivid 7 Dimension scanner (GE Vingmed Ultrasound, Horten, Norway) and the studies were both videotaped and stored digitally. Echocardiography at rest included standard views and measurements. Right atrial pressure at rest was estimated by inferior caval venous index,[20] and the following additional specific measurements of right ventricular performance[21–24] were also included:

- Tricuspid annular plane systolic excursion in millimetres measured in M-mode.[25]
- Tricuspid annulus peak systolic velocity in centimetres per second by colour tissue Doppler imaging and by pulsed tissue Doppler imaging.[26,27]

Approximately 1 hour after treadmill testing, exercise echocardiography was performed during supine cycling with about 30 degrees head elevation and 30 degrees left side tilt (Equipment: Ergoselect 1200 EL, Ergoline, Bitz, Germany). The stepwise World Health Organisation exercise protocol was used with a starting load of 25 Watt and an increase by 25 Watt every second minute until the target heart rate of $160^{-\mathrm{min}}$ was reached. Above that level, echocardiographic recordings become futile because of upper body movement and interposition of the

lungs. Systemic blood pressure was measured at every exercise level, as well as the maximal velocity of tricuspid regurgitation jet. The right ventricular systolic pressure was calculated from each recording by means of the modified Bernoulli equation, adding the right atrial pressure at rest to the calculated pressure gradient between right ventricle and right atrium (right ventricular systolic pressure $= 4\,V^2 + [\text{right atrial pressure}]$).[6]

In order to detect dynamic right ventricular outflow tract obstruction that could interfere with right ventricular systolic pressure measurements, an attempt was made to measure right ventricular outflow tract velocity at the 100 Watt level. Outflow tract velocities higher than 2 meters per second lead to exclusion from the study.

Data analysis

Pressure measurements were made offline by analysis of all digitally stored still images of tricuspid regurgitation velocity. Every frame was classified as a good, reasonable or poor/impossible measurement. For every minute of exercise, measurements were summarized into a conclusive pressure value (maximum of two values per workload level) based on the best accessible Doppler measurements. Obvious outlier measurements were ignored. For approval of the entire exercise study at least the second last passed workload level had to be evaluable.

For analysis of inter-observer variability, a blinded second analyser evaluated 35 exercise echocardiographic recordings from study participants and from patients with atrial or ventricular septal defects in a similar exercise study setting. The second analyser was experienced in exercise echocardiography technique. Inter-observer agreement was expressed by a Bland-Altman-plot[28] and by Kappa statistics.[29] Variability was also expressed as deviation (%) from the average of both analysers.

Data storage and statistical analysis were executed with Microsoft Access 2003, Microsoft Excel 2003, SPSS 12.1/16.0, Analyse-it 2.11 and SigmaPlot 11.0. Offline analyses of ultrasound studies were performed on EchoPac PC 5.x (GE Vingmed Ultrasound, Horten, Norway).

Statistics

Student t-tests were performed to compare different subgroups if normal distribution was assumed. Otherwise, nonparametric tests were used for continuous variables and Chi square for categorical variables. Pearson's correlation was used to determine relation between different independent variables. A multivariate linear regression model, using the stepwise backwards elimination procedure, was used to model the relation between independent variables, specifically age, gender, exercise habits, Z-score of peak oxygen uptake, tricuspid annular plane systolic excursion and tricuspid annular peak systolic velocity, and outcome variable maximal right ventricular systolic pressure during exercise. p-values lower than 0.05 were considered statistically significant. Inter-observer variability for continuous variables was calculated by intraclass correlation coefficient. For categorical variables, inter-observer agreement was calculated by kappa statistics.

Approvals

The study complies with the Declaration of Helsinki, and it was approved by the Regional Committee for Medical Research Ethics, with all participants giving their informed consent, minors by proxy. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

We recruited 113 healthy volunteers, who passed all examinations (Table 1).

Aerobic capacity characteristics

The aerobic capacity was normally distributed (Fig. 1), with a mean Z-score of 0.31 related to the reference study.[19] Of the individuals, 13 (11.5%), with 6 female, were athletes having Z-scores higher than 2.0, and 8 of these were aged from 16 to 18 years. The athletes performed different kinds of sports, including cross country skiing, soccer, badminton, volleyball, handball, and running, and they followed a training programme on a non-professional level beside their daily school or working activity.

Cardiac characteristics

We found 3 cases of right bundle branch block in the electrocardiogram at rest, 2 in normally trained subjects and 1 in an athlete, but no incidents of arrhythmia or bundle branch block occurring during exercise. All values for shortening fraction, right ventricular systolic pressure at rest, tricuspid annular plane systolic excursion, tricuspid annular peak systolic velocity and pulsed tricuspid annular peak systolic velocity were within normal limits and normally distributed (Table 2).

Exercise echocardiography/right ventricular systolic pressure characteristics

We obtained assessable echocardiography recordings in 109 individuals (96%), among whom 12 were athletes. Maximal right ventricular systolic pressure

Table 1. Demographics and basic characteristics.

Age group (years)	N	male/female	Age *Mean*	Height (cm) *Mean* ± *SD*	Weight (kg) *Mean* ± *SD*	BMI (kg/m^2) *Mean* ± *SD*
13–15	22	15/13	14.1	170.6 ± 11.9	62.0 ± 12.3	21.1 ± 2.6
16–18	19	22/19	16.9	168.9 ± 8.8	62.6 ± 11.6	21.8 ± 3.2
19–21	18	4/18	20.2	175.0 ± 8.6	68.3 ± 10.7	22.2 ± 2.1
22–25	21	8/14	23.3	171.8 ± 9.7	65.6 ± 17.1	22.0 ± 4.4
Overall/sum	113	49/64	18.1	171.1 ± 9.9	64.1 ± 12.9	21.8 ± 3.1

SD = standard deviation, BMI = body mass index.

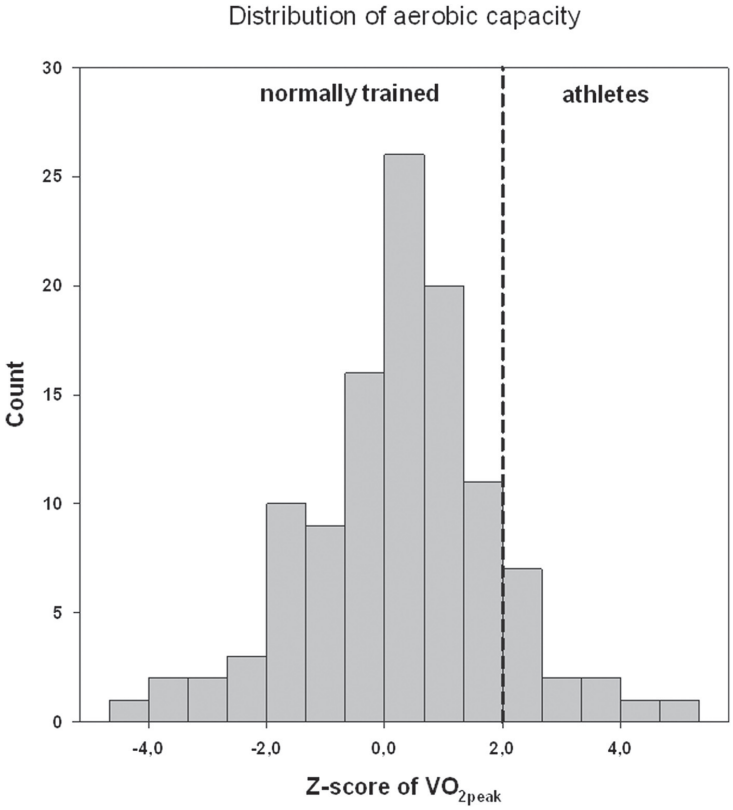

Figure 1.
Normal distribution of Z-score in peak oxygen uptake (n=113).

during exercise showed a skewed distribution towards high levels, while right ventricular systolic pressure in the normally trained group alone was normally distributed. For the whole group of 109 subjects, the maximal right ventricular systolic pressure during exercise showed a median of 39.0 millimetres of mercury, with a range from 17 to 69, and the 90th and 95th percentiles were 51 and 60 millimetres of mercury, respectively. In the normally trained group, the maximal right ventricular systolic pressure showed a mean of 38.0 millimetres of mercury, with standard deviation of 7.2, a median of 39.0, and a range from 17 to 63, with 90th and 95th percentiles at 46 and 51 millimetres of mercury. The athletes had a median maximal right ventricular systolic pressure of 55.5 millimetres of mercury, with a range from 28 to 69. The difference in maximal right ventricular systolic pressure during exercise between the normally trained group and the athletes was statistically

Table 2. Age related measurements.

Age group (years)	Z-score of VO_{2peak} *Mean ± SD*	Athletes *N*	FS (%) *Mean ± SD*	TAPSE (mm) *Mean ± SD*	TASM (cm/sec) *Mean ± SD*	Pulsed TASM (cm/sec) *Mean ± SD*	RVSP at rest (mmHg) *Mean ± SD*	Maximal RVSP (mmHg) *Median, range*
13–15	0.33 ± 1.70	2	32.9 ± 4.5	22.6 ± 2.6	10.51 ± 1.57	11.36 ± 1.57	21.8 ± 4.8	36.0, 28–60
16–18	0.62 ± 1.73	8	32.8 ± 4.1	22.7 ± 3.5	10.76 ± 1.76	11.84 ± 1.87	22.2 ± 3.8	41.0, 24–69
19–21	0.04 ± 1.27	2	31.8 ± 4.7	23.9 ± 3.1	10.59 ± 1.84	11.91 ± 1.95	21.6 ± 2.8	37.5, 17–57
22–25	0.02 ± 1.63	1	32.7 ± 3.8	23.5 ± 3.1	10.36 ± 1.22	11.36 ± 1.47	21.5 ± 3.2	39.9, 26–51
Overall/sum	0.31 ± 1.62	13	32.6 ± 4.24	23.1 ± 3.1	10.59 ± 1.62	11.64 ± 1.74	21.9 ± 3.8	39.0, 17–69

SD = standard deviation, FS = shortening fraction, TAPSE = tricuspid annular plane systolic excursion, TASM = tricuspid annular peak systolic velocity assessed by colour Tissue Doppler Imaging, pulsed TASM = pulsed Tissue Doppler measurements of TASM, RVSP = right ventricular systolic pressure.

Figure 2.
Maximal right ventricular systolic pressure (RVSP) during exercise in normally trained individuals and athletes. For normal trained group there are two outliers between 1.5 and 3 box lengths outside boxes edge, one extreme case outside > 3 box lengths.

significant, the Mann-Whitney test giving a value of p equal to 0.004 (Fig. 2).

In order to investigate whether low aerobic capacity could be caused by abnormal right ventricular systolic pressure, we compared the subgroup of 8 with lowest peak oxygen uptake, and Z-scores below −2.0, with the normal group having Z-scores between −2.0 and 2.0, but found no difference in maximal right ventricular systolic pressure. For the entire normally trained group, there was no correlation between peak oxygen uptake and maximal right ventricular systolic pressure.

Normal and abnormal responders in terms of right ventricular systolic pressure, taking a cut-off for maximal right ventricular systolic pressure of greater than 50 millimetres of mercury, showed a similar pattern of slow and parallel rise of right ventricular systolic pressure and systemic systolic blood pressure during incremental exercise (Figs 3 and 4). Normal responders reached a plateau at a moderate level for exercise, whereas abnormal responders showed a continuous increase of systolic pressure throughout the entire duration of exercise (Fig. 3).

Of the 12 athletes, 10 (83%) had maximal right ventricular systolic pressures higher than 40 millimetres of mercury, as compared to 44 of the 97 (45%)

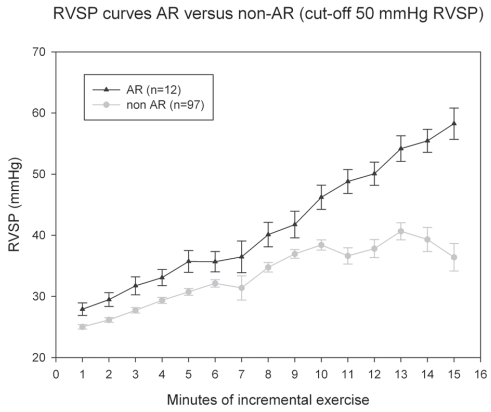

Figure 3.
Right ventricular systolic pressure (RVSP) during exercise in subjects with abnormal right ventricular systolic pressure response (AR) and normal response (non-AR) with cut-off right ventricular systolic pressure 50 mmHg. After 15 minutes, there were too few measurements to permit calculation of confidence intervals.

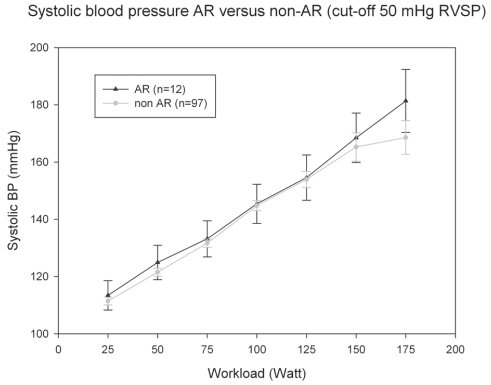

Figure 4.
Systolic blood pressure during exercise in subjects with abnormal right ventricular systolic pressure response (AR) and normal response (non-AR) with cut-off right ventricular systolic pressure 50 mmHg. After 175 Watt workload, there were too few measurements for calculation of confidence intervals.

normally trained subjects ($p = 0.025$). When using 45 millimetres of mercury as the cut off, the respective number of abnormal responders were 8 of 12 (67%) for athletes, and 16 of 97 (16%) for normally trained subjects ($p < 0.001$). With 50 millimetres of mercury taken as the cut-off, the respective numbers were again 8 of 12 (67%) for the athletes, but only 8 of 97 (8%) of the normally trained subjects ($p < 0.001$) (Table 3).

Linear regression analysis showed that age, gender, body mass index, exercise habits, Z-score of peak oxygen uptake, tricuspid annular plane systolic excursion and tricuspid annular peak systolic velocity did not have a significant predictive value for maximal right ventricular systolic pressure during exercise.

There was no difference in maximal right ventricular systolic pressure between 9 smokers and 98 non-smokers. For females there was no difference in maximal right ventricular systolic pressure between groups with regard to use of oral contraceptives.

Inter-observer variability

The analyses made by the 2 independent observers showed very good agreement. The 95% limits of agreement were −0.17/0.33 metres per second, with a positive bias of 0.06 metres per second in Doppler velocity measurements and 2 millimetres of mercury in maximal right ventricular systolic pressure between the first and second analyser (Fig. 5). There was no difference in agreement depending on low or high velocities. For tricuspid regurgitation velocities measured offline by the two observers, the intraclass correlation coefficient was 0.934 ($p < 0.001$).

In order to define abnormal pressure response to exercise, it was possible to measure inter-observer agreement for a nominal variable by Kappa statistics. With a cut-off of 45 millimetres of mercury, Kappa was 0.79, while with a cut-off of 50 millimetres of mercury, Kappa was 0.82. Following commonly used definitions, kappa from 0.6 to 0.8 means good agreement, while kappa above 0.8 means very good agreement.

Discussion

Our data has shown a great variability in right ventricular pressure response to exercise. There is no linear correlation between maximal right ventricular systolic pressure and aerobic capacity, but athletes with high aerobic capacity often show an abnormally high response. The upper normal limit of the response in normally trained individuals seems to be higher than commonly assumed.[9]

The number of examined individuals in our study is high compared to other studies with a comparable focus that investigated mixed groups of normally trained subjects and athletes made up of 20[3] to 40 individuals.[1] Our study group has a sufficient distribution of age and gender to allow conclusions to be drawn concerning adolescents and young adults. This is also supported by results for peak uptake of oxygen, which reflect reference values.

The strong correlation between non-invasive and invasive measurements has previously been

Table 3. Abnormal right ventricular systolic pressure response during exercise in normally trained group and athletes.

Study group	N	Cut-off 40 mmHg N (%)	Cut-off 45 mmHg N (%)	Cut-off 50 mmHg N (%)
Normally trained group	97	44 (45%)	16 (16%)	8 (8%)
Athletes	12	10 (83%)	8 (67%)	8 (67%)
Chi square significance (p)		0.025	<0.001	<0.001

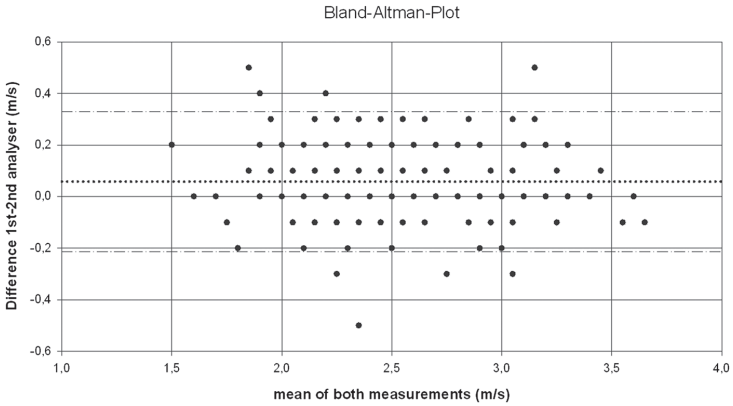

Figure 5.
Bland-Altman-Plot of inter-observer variability in measurements of the velocity of the jet of tricuspid regurgitation.

demonstrated both at rest and during exercise.[6,7,20] Thus conclusions about the response of pulmonary arterial pressure can rely, therefore, on entirely non-invasive protocols, albeit that normal values for exercise echocardiography have not previously been established. Our findings, nonetheless, confirm earlier statements[7] that exercise echocardiography as a reliable tool for assessment of the response of right ventricular pressure during exercise.

The high rate of quantifiable tests as compared to previous studies,[30] and a high degree of inter-observer agreement, reflects the technical progress. Modern ultrasound scanners and digital storage of image information facilitate detection and precise determination of small tricuspid regurgitation signals.

Doppler signal profiles of tricuspid regurgitation velocities during exercise are of variable quality, and not always easy to interpret. Signal drop-out often occurs in the middle of the profile. Measurement of the angle of velocity may be disturbed during exercise because of increased translation movement of the heart and narrowing of the area of good ultrasonic access with increasing excursion of the thorax. The described non-invasive method includes the assumption that right atrial pressure remains unchanged during exercise. From invasive studies,[7] we know that this assumption may not be totally correct, leading to a slight underestimation of pulmonary arterial pressure during exercise.

All of these technical issues potentially cause a certain underestimation of the response of right ventricular systolic pressure, and none would cause overestimation. The low inter-observer-variation confirms the high accuracy in pressure measurements.

Thus, in the absence of any obstruction to right ventricular outflow, our current non-invasive data allows conclusions to be drawn about pulmonary arterial pressure and pulmonary vascular resistance, though these parameters had not been measured. And pulmonary hemodynamics during exercise are in focus when seeking early signs of pulmonary vasculopathy.

In our study, we performed exercise echocardiography 1 hour after a maximal treadmill exercise test. This may have lowered pulmonary and systemic resistance, resulting in false low levels of pulmonary pressure. For that reason, as well as technical ones, our protocol may have caused underestimation of the response of pulmonary pressure. There are several possible ways, therefore,

of underestimating pulmonary arterial systolic pressure by non-invasive measurement of right ventricular systolic pressure, but no obvious risk of overestimating it.

The normal range of right ventricular systolic pressure during exercise in our material is wider than in previous studies. The Gaussian distribution of maximal right ventricular systolic pressure in normally trained subjects confirms a high variability of pressure response as a physiological phenomenon rather than a technical or methodical artefact.

The data we have presented confirms a strong interrelation between high aerobic capacity and an abnormal response of right ventricular systolic pressure. The fact that separation of athletes from the normal trained group lead to normal distribution in maximal right ventricular systolic pressure during exercise suggests a specific physiological mechanism in athletes leading to high right ventricular and pulmonary pressure during exercise. This is also supported by the significant difference in the abnormal response between normal trained individuals and athletes. Cardiac output, alveolar gas diffusion and pulmonary vascular resistance were not monitored during exercise. Thus, our data does not allow a conclusion to be drawn about the theory that elevated pulmonary arterial pressure in athletes is caused by high cardiac output and limited dilative capacity on the pulmonary vessels.[1,15] The data did not give any evidence that high right ventricular systolic pressure during exercise reduces aerobic capacity in healthy individuals, since there was no difference in right ventricular systolic pressure between normally trained and totally untrained individuals.

Like earlier studies[1,15] we found that normally responding individuals reach a plateau in right ventricular systolic pressure, whereas individuals with an abnormal response continue to increase both their right ventricular and pulmonary arterial systolic pressures. There was no sudden increase of pulmonary or systemic vascular resistance in individuals with an abnormal response. The continuous increase of right ventricular systolic pressure in individuals showing an abnormal response cannot be explained by recent theories of sympathoexcitation and its influence on vasoconstriction in the pulmonary arterioles.[31] Because of their different physiology, we had to exclude the athletes in order to define normality in right ventricular systolic pressure. This leads to an upper limit of 50 millimetres of mercury for the normal response to exercise in healthy normally trained young individuals. Given that right ventricular systolic pressure reflects pulmonary arterial systolic pressure, 50 millimetres of mercury pulmonary arterial systolic pressure calculated into mean pulmonary arterial pressure results in 32.5 millimetres of mercury when using the formula $(PAMP = 0.61 * PASP + 2\,mmHg)m^4$ where PAMP and PASP are the mean and systolic pulmonary arterial pressure respectively. The result of 32.5 fits with the definition of exercise-induced pulmonary hypertension as mean pulmonary arterial pressure higher than 30 millimetres of mercury during exercise.[5]

Our results challenge the commonly used value of 40 or 45 millimetres of mercury as the upper limit of the normal response of right ventricular systolic pressure to exercise. The acceptance of a higher upper limit for normal pressure may lead to a more restrictive definition and reduced prevalence of exercise-induced pulmonary arterial hypertension.

In conclusion, we have shown exercise echocardiography to be a reliable tool in measuring right ventricular systolic pressure during exercise. Our study demonstrates a high variability in the response of this pressure to exercise among adolescents and young adults. According to our findings, 50 millimetres of mercury is the upper limit of the normal response to exercise in healthy normally trained subjects. The common definition of the normal range in right ventricular and pulmonary arterial pressures during exercise may have to be reconsidered.

Young non-professional endurance-trained athletes show an abnormally high response during exercise, with two-thirds exceeding 50 millimetres of mercury. This has to be taken in account in the selection of controls in clinical studies of exercise-induced pulmonary hypertension

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II

III

IV

A novel BMPR-2 gene mutation associated with exercise-induced pulmonary hypertension in septal defects

Short title: BMPR-2 gene mutations in septal defects

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Abstract:

Objective: Our study aimed to investigate the relationship between exercise-induced pulmonary arterial hypertension and genetic changes related to the transforming growth factor-β (TGF-β) signaling pathway in patients with cardiac septal defects.

Design: In a population-based group of 44 patients (age 13 – 25 years) with either isolated ventricular septal defect (n=27) or isolated atrial septal defect (n=17), right ventricular systolic pressure response to submaximal exercise was studied by echocardiography and classified as normal (≤ 45 mmHg), borderline (45-50 mmHg) or abnormal (>50 mmHg). Genes related to TGF-β were analyzed by DNA sequencing and multiplex ligand-dependent probe amplification. None of the patients were related.

Results: Pressure response was borderline in 5 and abnormal in 9 patients. Five patients showed mutations in exon 12 of the bone morphogenetic protein receptor type 2 gene: 3 patients with normal pressure response were heterozygous for the benign mutation S775N (c. 2324, G>A), 2 patients with borderline/abnormal pressure response were heterozygous for the probably pathogenic mutation Y589C (c.1766, A>G) which has not been previously described.

Conclusion: Genetic changes in TGF-β related genes may be overrepresented in patients with cardiac septal defects and exercise-induced pulmonary hypertension.

Abbreviations:

ALK1	activin receptor-like kinase 1
ASD	atrial septal defect
BMPR2	bone morphogenetic protein receptor type 2
FPAH	familial pulmonary arterial hypertension
MLPA	multiplex ligation-dependent probe amplification
PAH	pulmonary arterial hypertension
RVSP	right ventricular systolic pressure
TGF-beta	transforming growth factor-β
VSD	ventricular septal defect

Introduction:

Proteins of the transforming growth factor-β (TGF-β) signaling pathway have been shown to be important prenatally in cardiac and pulmonary vascular development (1-4) as well as in the postnatal pathogenesis of pulmonary arterial hypertension (PAH).(5-8) Bone morphogenetic protein type 2 (BMP2) is crucial in coordinating multiple aspects of atrioventricular canal morphogenesis (4) and cardiac looping.(1) Mutations in the bone morphogenetic protein receptor type 2 (BMPR-2) coding gene have been identified in familial PAH (FPAH) (6) and in about 25% of sporadic cases of idiopathic PAH.(9) In a mixed group of patients with congenital heart disease and secondary PAH, BMPR-2 gene mutations have been demonstrated in 6% of cases.(10) In patients with atrial septal defect (ASD) with or without Eisenmenger syndrome no mutations were found.(11) In patients with Hereditary Hemorrhagic Teleangiectasia (Osler-Weber-Rendu syndrome) and associated PAH, mutations of two other TGF-β genes have been identified: activin receptor-like kinase 1 (ALK1) (12) and endoglin (ENG).(13)

Occurrence of secondary PAH with small open septal defects and even many years after surgical defect closure, has been reported in patients without indications of perioperative PAH.(14;15) Echocardiography has been shown to give reliable measurements of right ventricular systolic pressure (RVSP) compared to invasive measurement at rest (16) and during exercise.(17) In the absence of right ventricular outflow tract obstruction, RVSP reflects pulmonary arterial systolic pressure.(18-20)

The role of genetics in patients with congenital heart disease and secondary PAH has yet to be defined. We have found exercise-induced PAH in 25% of adolescents with cardiac septal

defects.(21) We aimed to investigate the relationship between exercise-induced PAH in this group of patients and genetic changes related to the TGF-β signaling pathway. The hypothesis is that genetic changes would predispose to exercise-induced PAH.

Material and methods:

According to medical registries in the Norwegian regions of Vestfold, Asker and Bærum, 69 children were born between 1982 and 1993 with isolated ventricular septal defect (VSD) or isolated ASD. Exclusion criteria were right ventricular outflow tract obstruction, spontaneous defect closure during follow-up and complicating conditions preventing appropriate exercise testing (i.e. trisomy 21). Two individuals with device-closed ASD were excluded to prevent device related confounding. The 67 eligible patients had at the time of inclusion either an open defect or they had undergone surgical defect closure. Twenty-three patients were lost to follow-up or refused participation. Consequently, a population-based group of 44 individuals (age 13 – 25 years) with isolated ASD (n=17) or isolated VSD (n=27) could be included. There were no family relationships between any of the patients. A control group for genetical results was not included; a comparison between the patient group and a matched control group in terms of baseline and exercise hemodynamic results has been described previously.(21)

Hemodynamic assessment

Echocardiographic recordings were obtained with a Vivid 7 scanner (GE Vingmed Ultrasound, Horten, Norway). All echocardiographic studies were videotaped and saved digitally (still frame and loops). Right atrial pressure at rest was estimated by vena cava

inferior index.(22) Exercise echocardiography was performed during supine cycling with about 30 degrees elevation and left side tilt (Equipment: Ergoselect 1200 EL, Ergoline GmbH, Bitz, Germany). A stepwise exercise protocol was used with a starting load of 25 Watt and an increase of 25 Watt every second minute until the target heart rate of 160^{-min} was reached. Above that level, echocardiographic recordings become futile because of upper body movement and interposition of the lungs. The maximal velocity of tricuspid regurgitation jet was measured at every exercise level. The RVSP was calculated from each recording by means of the modified Bernoulli's equation, adding the right atrial pressure at rest to the calculated pressure gradient between right ventricle and right atrium.(16) In order to detect dynamic right ventricular outflow tract obstruction that could interfere with RVSP measurements, right ventricular outflow tract velocity was measured at the 100 Watt level. Patients with outflow tract velocities higher than two meters per second were not included in the study. Maximal RVSP from 46 mmHg through 50 mmHg during exercise was defined as borderline, maximal RVSP exceeding 50 mmHg was defined as abnormal.(23)

Pressure measurements were made offline by analysis of all digitally stored still frames of tricuspid regurgitation velocity. Offline analysis of ultrasound studies were performed on EchoPac PC 5.x (GE Vingmed Ultrasound, Horten, Norway). Every frame was classified into good, reasonable or not analyzable measurement. For every minute of exercise, measurements were summarized into a conclusive pressure value (maximum of two values per workload level) based on the best accessible Doppler measurements. Obvious outlier measurements with major deviation from both previous and subsequent measurements were rejected. For approval of the entire exercise study at least the second last passed workload level had to be evaluable.

Genetical analysis

Screening for mutations in the BMPR-2 gene (GenBank accession number Z48923.1) was performed by DNA sequencing to detect point mutations and small insertions/deletions within exons and the flanking intron sequences, and by multiplex ligation-dependent probe amplification (MLPA) to detect structural alterations. For DNA sequencing, DNA was extracted from EDTA-containing blood by the use of a BioRobot EZ1 (Qiagen GmbH, Hilden, Germany). Individual exons with flanking intron sequences were amplified by PCR. Typically, approximately 50 base pairs of flanking intron sequences were included in the amplicons. The primer sequences and conditions for the thermal cyclings are available upon request. Standard DNA sequencing reactions using version 3.1 of Big Dye terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) were analyzed on a Genetic Analyzer 3730 (Applied Biosystems Inc., Foster City, CA). The software prediction program PolyPhen (www.bork.embl-heidelberg.de/PolyPhen/) was used to assess the pathogenicity of identified missense mutations. For MLPA analysis, DNA was extracted from EDTA-containing white blood lymphocytes by the manufacturer's protocol, on a Magnapure DNA Extractor (Roche Diagnostics GmbH, Mannheim, Germany). The SALSA MLPA kit P093 HHT/PPHT1 (MCR-Holland, Amsterdam, The Netherlands) was used to test for deletions or duplication in the BMRP-2 gene, the ENG gene and the ALK1 gene.

The study complies with the Declaration of Helsinki. It was approved by the Regional Committee for Medical Research Ethics and all participating subjects gave informed consent. The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results:

The study included 44 patients (17 ASD, 27 VSD) (table 1). All patients with ASD had a surgically closed defect. Sixteen VSD patients were not operated and had a small open septal defect (either muscular or perimembraneous). Velocity measurements across the ventricular septum in these patients revealed high pressure gradients (4.5±0.51 m/s) indicating normal systolic pressure conditions in both heart chambers at rest. Eleven patients had their VSD closed surgically. No patient had a history of preoperative PAH. Two of the operated patients with VSD (18%) had minor residual shunts with normal pressure conditions at rest. Median age at the time of defect closure was 53 months for ASD patients and 61 months for VSD patients. None of the patients had family members with congenital heart disease or known PAH within two generations.

There was no case of pulmonary hypertension at rest (RVSP > 40 mmHg). Exercise echocardiography permitted assessable measurements of RVSP by tricuspid regurgitation jet velocity in all patients. Five patients showed borderline RVSP and 9 patients had abnormal RVSP during exercise (table 2).

Two subjects were found to be heterozygous for mutation Y589C (c.1766, A>G) in exon 12 of the BMPR-2 gene and three were found to be heterozygous for mutation S775N (c. 2324, G>A) in exon 12 (table 2). Analysis of the pathogenicity of two mutations by the use of the prediction program PolyPhen, revealed that mutation Y589C was predicted to be “Probably damaging”. S775N was predicted to be “Benign” and has been found in healthy controls subjects by other investigators.(24) None of the subjects had deletions or duplication in the BMPR-2 gene, the ENG gene or the ALK1 gene.

Discussion:

Our study demonstrates the presence of genetic changes related the TGF-β signaling pathway in patients with cardiac septal defect and borderline or abnormal RVSP response to submaximal exercise. To our knowledge mutation Y589C in the BMPR-2 gene is a novel mutation predicted by PolyPhen to be pathogenic, whereas mutation S775N has been previously reported (24) and has also been found in healthy controls.(25)

Exercise-induced PAH characterized by abnormal RVSP increase during exercise is considered being a marker of early pulmonary vasculopathy in FPAH.(26;27) Detection of exercise-induced PAH identifies carriers of BMPR-2 gene mutations in FPAH.(26;28) An animal study has demonstrated that targeted genetic ablation of the BMPR-2 gene in the pulmonary vascular endothelium predisposes to PAH. Congenital heart malformations with left-to-right shunt like ASD and VSD lead to volume overload or combined pressure and volume overload in the pulmonary vascular system. Pressure and volume cause endothelial stretch that in turn leads to pulmonary vasculopathy and eventually results in PAH.(29) Our data support the hypothesis of genetical susceptibility to PAH when the pulmonary vascular endothelium is exposed to abnormal hemodynamics previous to closure of significant septal defects.

The carrier frequency of pathogenic mutations of BMPR-2 has been estimated to be 0.01-0.001% in the healthy normal population.(30) This low carrier frequency in the normal population makes it obsolete to calculate a statistical significance of several cases of mutations in a minor study group like this. Thus, our finding of two independent cases of

BMPR-2 mutation among 44 patients probably must be considered important for either exercise-induced PAH or congenital cardiac septal defects or for both. The TGF-β superfamily plays an essential role in cardiac and pulmonary vascular development as well as in the pathogenesis of PAH. Consequently, the presence of a congenital cardiac septal defect and the presence of secondary PAH may be two different expressions of the same genetically determined general vasculopathy. The long-term implications of exercise-induced PAH in congenital heart disease are unknown.(27) Genetical changes may increase the risk of deterioration into PAH at rest. Thus, genetical analysis in all children with congenital septal defects may facilitate that mutation carriers will have their defect closed early in life with subsequent life-long medical follow-up.

In conclusion we have shown that genetic changes in TGF-β related genes may be overrepresented in patients with cardiac septal defects and exercise-induced pulmonary hypertension. The mutation Y589C in the BMPR-2 gene has not previously been reported.

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Tables

Table 1: Basic demographics

N	**Sex**	**Age (years)**	**Height (cm)**	**Weight (kg)**	**BMI[2](kg/m^2)**
	(male/female)	*Mean±SD*[1]	*Mean±SD*	*Mean±SD*	*Mean±SD*
44	**19/25**	**17.5±3.3**	**167.4±8.8**	**59.7±11.0**	**21.3±3.5**

[1] : SD = standard deviation

[2] : BMI = body mass index

Table 2--Hemodynamic and genetic results

Patient No.	Sex	Age (years)	Diagnose	Maximal RVSP during exercise (mmHg)	Mutation / deletion
1	female	19	untreated VSD	24	-
2	female	19	closed ASD	24	-
3	male	24	closed VSD	24	-
4	female	18	closed ASD	26	-
5	male	15	closed ASD	28	**S775N**[1]
6	female	20	closed VSD	28	-
7	female	21	untreated VSD	30	-
8	female	14	untreated VSD	30	-
9	female	15	closed VSD	30	-
10	female	17	untreated VSD	32	-
11	male	14	untreated VSD	32	-
12	female	16	closed ASD	34	-
13	male	15	closed ASD	34	**S775N**[1]
14	female	23	closed ASD	34	-
15	female	23	closed ASD	34	-
16	female	16	closed ASD	34	-
17	female	20	closed ASD	36	-
18	male	15	closed VSD	36	-
19	male	19	untreated VSD	36	-
20	female	13	untreated VSD	36	-
21	male	18	closed VSD	36	-
22	male	16	closed ASD	36	-
23	female	17	closed ASD	38	-
24	female	15	closed ASD	39	-
25	female	21	untreated VSD	39	-
26	male	14	untreated VSD	41	-
27	female	17	untreated VSD	41	-
28	female	18	closed ASD	41	-
29	male	16	untreated VSD	41	**S775N**[1]
30	male	21	closed ASD	43	-
31	female	13	closed VSD	46	-
32	male	20	closed ASD	46	-
33	female	21	untreated VSD	49	-
34	female	18	closed ASD	49	**Y589C**[2]
35	female	16	closed ASD	49	-
36	male	16	untreated VSD	51	-
37	female	24	closed VSD	54	-
38	male	12	untreated VSD	57	-
39	male	15	closed VSD	57	**Y589C**[2]
40	male	17	untreated VSD	57	-
41	female	12	closed VSD	61	-
42	male	22	untreated VSD	73	-
43	male	13	closed VSD	73	-
44	male	21	closed VSD	76	-

The patients are sorted in ascending order by maximal RVSP during submaximal exercise.
[1] : Mutation S775N (c. 2324, G>A) in exon 12 of the BMPR-2 gene
[2] : Mutation Y589C (c.1766, A>G) in exon 12 of the BMPR-2 gene